Complex Assessment of Functional Activity of Chang Liver Cell Culture in Blood Serum of Patients with Liver Diseases of Various Etiology

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Immortalized cell culture of hepatocytes, Chang liver, is one of the candidates for the use in "bioartificial liver" systems.

The aim of the investigation was to evaluate the possibility of using the Chang liver cell culture as a bioreactor cell kit making a complex study of biochemical parameters of its effect on the blood serum of patients with liver diseases of various etiology.

Materials and Methods. Samples of blood serum from two groups of patients were investigated: patients with obstructive jaundice were included in group 1 "jaundice" (n=9), group 2 "cirrhosis" (n=10) comprised patients with hepatic cirrhosis and hepatocellular jaundice. To study the effect of the cultured Chang liver cells on the patients' blood serum, confluent monolayer of the cells was incubated with serum samples at 37°C in a 5% CO_2 atmosphere with the ratio of $(2.0–2.1)\cdot10^5$ cells per 0.105 ml of serum during 12 h. On completion of the process, the values of the main biochemical parameters of synthetic (albumin, urea, transthyretin) and detoxifying (total bilirubin fraction) functions were determined, as well as markers of cell destruction (hepatic transaminases, lactate dehydrogenases). Viability cell changes after exposure to the serum were defined by MTT test.

Results. It was estimated, that complex assessment of biochemical parameters of synthetic and detoxifying functions of the Chang liver culture relative to the blood serum of the patients of both groups, is the most informative one. This cell culture is synthetically active to the most extent in respect to the serum of the patients in "cirrhosis" group, while in "jaundice" group the reduction of this biochemical parameter was observed. Detoxifying activity of the Chang liver culture, exhibited in the dynamics of bilirubin fractures, was noted in both groups, but in "cirrhosis" group it was most marked, as it occurred in half the cases. Biochemical components of the serum, in their turn, also influenced cell viability. Serum samples of both patient groups were found to inhibit the viability of the cells almost in 50% of cases for each group.

Conclusion. The results of complex assessment can be used to determine the efficacy of applying various cell cultures as a model system in the development of "bioartificial liver" systems. Chang liver cell culture, according to the complex assessment results, is most active relative to the blood serum of the "cirrhosis" group of patients with impairments of synthetic and detoxifying liver functions.

Key words: Chang liver cell culture; bioartificial liver; hepatic failure.

At present, the most effective method of treating hepatic failure is liver transplantation: either of the whole organ from the postmortem donor or its fragment from the living relative or postmortem donor, suggesting its future hypertrophy in the post-operative period. However, the number of operations for liver transplantations is limited in our country and abroad: every third patient dies while on the Waiting List to receive a donor liver, mainly because of the organ scarcity and a long-term period of seeking a compatible donor [1]. To reduce the mortality rate among the patients with liver insufficiency, effective extracorporeal systems are being developed to maintain the liver functions till the time of transplantation, which are capable to perform not only detoxifying [1, 2], but metabolic, synthetic and regulatory functions as well [3, 4]. In this connection, since 80-ies of the last century supporting systems, combining functions of blood/plasma perfusion and cell technologies, have been worked out.

Liver support systems using live hepatocyte cultures — "artificial liver" bioreactors — have been developed. Cultures of hepatocyte-like cells in these

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systems enrich patient's plasma and blood with the synthesis products (albumin, bile acids, clotting factors and so on) and are capable of partial detoxification. In order to perform effectively bioregulatory and synthetic functions cells must meet several criteria: to perform the functions of a normal liver hepatocyte (detoxification, synthesis of biologically active substances), actively proliferate (accumulating minimum 400 g of biomass volume) in the composition of bioreactor, function in the continuous contact with the plasma of patients having acute liver failure [5].

There are several approaches to creation of optimally acting cell lines in the composition of bioreactors, of which application of immortalized and genetically modified lines of animal and human hepatocytes is recognized to be most promising [6]. These cells possess unlimited but controlled capability to division and preserve the main biological characteristics and functions of primary hepatocytes [7]. Various immortalized cell lines of hepatocytes are used: PICM-19 line from 8-day-old porcine embryo [8], human immortalized lines of hepatocyte-like cells HepZ [9], HepG2 [10], cBAL111 [11]. Chang liver line, created in 1954 presumably by contamination of normal hepatocytes by the cells of HeLa line, has been also used [12]. Investigators are of different opinion on the possibility and permissibility of its application as a model of the normal hepatocyte because of its oncogenic potential [13], however, a sufficient number of research groups, engaged in the problem of making "bioartificial liver" bioreactors, consider the Chang liver line to be one of the candidates of using it in the composition of artificial systems [14].

More than two decades have passed since the advent of the first bioreactors. Many technical solutions have been found, approaches to the cell culturing improved. There have been designed systems, which successfully undergone clinical trials, and one of them ELAD system (Vital Therapies, Inc., USA) is already being used in the clinic [15]. But plenty of problems in the field of cell technologies remained unsolved, including those connected with the increase of life time of hepatocyte cultures. To prolong the functional life of hepatocytes, investigations are being carried on to create bioactive matrixes, to seek for new engineering solutions to provide the cells with oxygen and all the necessary substances.

The aim of the investigation was to evaluate the possibility of using Chang liver cell culture as a cell kit for a bioreactor by a complex study of biochemical parameters of its effect on the blood serum of patients with hepatic diseases of various etiology.

Materials and Methods. Chang liver cell line (ATCC®CCL-13™; Scientific Research Institute of Virology of Russian Academy of Medical Sciences, Russia) was used in the work. The cells were cultured in DMEM medium (PanEco, Russia), containing 10% of fetal bovine serum (HyClone, USA) and 2 mM of L-glutamine (PanEco, Russia). The cells were cultured in

CO_2-incubator at 37°C and at 5% CO_2, at every passage stage the cells being treated with 0.25% trypsin-EDTA (PanEco, Russia). Cells were counted by the standard method using Goryayev chamber.

Cell viability was defined by MTT test, based on the capability of mitochondrial dehydrogenases to convert water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). MTT matrix solution (in the concentration of 5 mg/ml in incomplete cell medium DMEM) was prepared for this purpose. The obtained solution was added to the wells of 96-well plate in the ratio of 1:10 relative to the volume of the growth medium, and incubated for 3 h at 37°C in CO_2-incubator. Then liquid was removed, 100 µl of DMSO (dimethyl sulfoxide) was added to each well with its following pipetting for receiving uniform dyeing. Optical density was calculated then with a plate reader EMax Plus (Molecular Devices, USA), using 540–590 nm filter as the main and 630–690 nm filter as a correcting one. Blood for serum was collected from the patients with the help of a vacuum system S-Monovette (Sarstedt, Germany), mixed with anticoagulant, and after the completion of the clotting process the clot was centrifuged (10 min, 3000 rev/min), and supernatant fraction was used for the experiments. Blood sampling, serum preparation, storage and determination of biochemical parameters were made in the clinical laboratory of the Volga District Medical Centre under Federal Medical and Biological Agency of Russia. Biochemical parameters of blood serum were determined using automatic analyzer KONELAB 20 (Thermo Electron, Finland), and diagnostic kits (Analyticon Biotechnologies AG, Germany) for determination of albumin, urea, aspartate aminotransferase (AcAT), alanine aminotransferase (AlAT), lactate dehydrogenase (LDG), bilirubin fractions, and a kit for transthyretin determination (Thermo Fisher Scientific, Finland).

Blood serum samples were examined for two groups of patients with hepatic ailments of various etiology. They were collected once before treatment of the main disease. Nine patients (5 males and 4 females) with clinical and laboratory manifestations of obstructive jaundice caused by mechanical obstruction of extrahepatic ducts (cancer of the pancreas head, metastatic lesion of the liver portal area, choledocholithiasis) were included in the group "jaundice". The average age of the patients was 60 years, the disease lasted 1–3 months. Ten patients (5 males and 5 females) with hepatic cirrhosis of different etiology composed the "cirrhosis" group. Serum specimens were taken when patients were placed in a Waiting List for liver transplantation. Mean age of the patients was 42 years with the disease duration from 8 months to 18 years (4 years on average). Enumeration of serum samples (P-1–P-20) was done in the order of their delivery for experiments, therefore enumeration "breakdown" in the groups took place. Serum sample P-9 from the group "jaundice" was excluded due to the violation of cryostorage conditions. The main biochemical

T a b l e 1

The main biochemical parameters of blood serum of the patients with liver diseases

| Parameters	Norm	Group "jaundice" (n=9)	Group "cirrhosis" (n=10)
Albumine (g/L)	35.0–52.0	24.6–52.3	15.6–38.3
Transthyretin (g/L)	0.2–0.4	0.0–0.3	0.1–0.2
Urea (mmol/L)	1.7–8.3	2.0–20.9	1.9–17.4
Total bilirubin (μmol/L)	5.0–21.0	62.9–1062.7	16.5–653.4
Conjugated bilirubin (μmol/L)	0.0–7.0	30.4–603.3	5.9–307.5
Unconjugated bilirubin (μmol/L)	5.0–14.0	10.7–526.5	10.9–292.2

parameters of the patient blood serum in both groups are presented in Table 1.

The study complies with the Declaration of Helsinki (the Declaration was passed in Helsinki, Finland, June, 1964, and revised in October, 2000, Edinburg, Scotland) and was performed following approval by the Ethic Committee of Nizhny Novgorod State Medical Academy. Written informed consent was obtained from every patient.

To study the effect of the Chang liver cell culture on the patients' blood serum, confluent monolayer of the cells was incubated with the serum samples at 37°C and in a 5% CO_2 atmosphere with the ratio of $(2.0–2.1)\cdot10^5$ cells per 0.105 ml of serum during 12 h. The maximum time of incubation was chosen for the cells not to lose their metabolic functions [16].

On completion of the process, the values of the main biochemical parameters of synthetic and detoxifying functions were determined, as well as of the markers of cell destruction. Viability cell changes after exposure to the serum were defined by MTT test.

Synthetic activity of hepatocytes was defined by the increase of albumin (g/L), urea (mmol/L), and transthyretin (g/L) content.
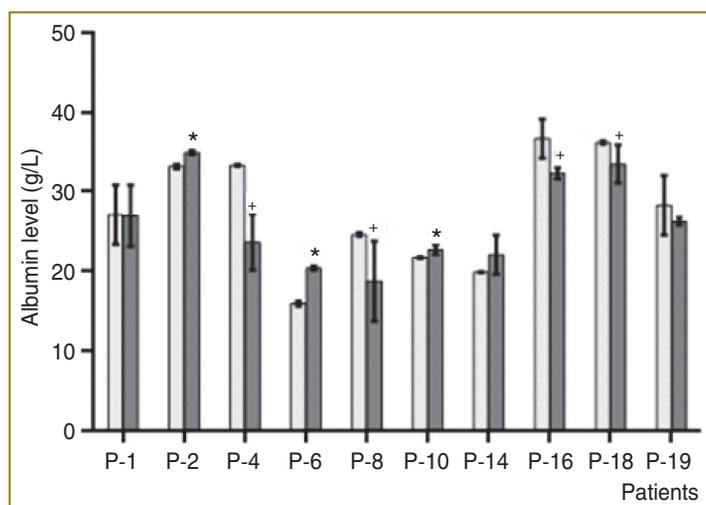
Detoxifying hepacyte activity was detected by total bilirubin content (μmol/L), and by its fraction dynamics: conjugated and indirect (unconjugated) bi-lirubin. Direct-reacting bilirubin is bilirubin-glucuronide, which is called conjugated bilirubin, as its free molecules are conjugated with glucuronide radical in hepaticytes. The resulting complex compound is soluble in water and is normally excreted through the bile ducts. Indirect-reacting bilirubin is un-conjugated bilirubin as it is not bound to glucuronide in the liver. In plasma, this type of bilirubin forms a complex compound with albumin. Thus, decrease of the total bilirubin together with the increase of conjugated bilirubin and reduction of uncojugated bilirubin in the blood serum after the exposure to the cell culture are indicative of an active detoxifying work of hepatocytes.

Cytotoxicity of the serum relative to the cell cultures was determined by the changes of the content of the cell destruction markers — AcAT (units/L), AlAT (units/L), LDG (units/L). A negative effect of the serum on the cells resulted in the increase of their values, and decrease of values or insignificant changes spoke of the absence of any effect on the cell life activity.

The experimental data were statistically processed using Microsoft Excel and Graf Pad software. The results are presented as M±σ (SD). Statistical significance of the mean differences was determined by Mann–Whitney criterion.

Results. Statistically significant rise of the albumin level was found to occur in three serum samples of the "cirrhosis" group (P-2, P-6, and P-10), but the normal value of albumin was not reached in any case (Figure 1). Elevation of the urea level was noted in samples P-10, P-16, and P-18, and the level of transthyretin

Figure 1. Change of the albumin level in the blood serum of patients from the group "cirrhosis" after incubation with the cell culture (light field — before exposure, dark field — after exposure); * statistically significant rise of the albumin level relative to the initial value in a given patient; + statistically significant decrease of the albumin level relative to the initial value in a given patient

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T a b l e 2

Changes of biochemical parameters of synthesis in the patients' blood serum after incubation with the cell culture

Parameters	Group "jaundice"									Group "cirrhosis"									
	P-3	P-5	P-7	P-11	P-12	P-13	P-15	P-17	P-20	P-1	P-2	P-4	P-6	P-8	P-10	P-14	P-16	P-18	P-19
Albumin	↓	↓	↓	↓	↓		↓			↑	↓	↑	↓	↑			↓	↓	
Urea					↓						↓	↓		↑			↑	↑	
Transthyretin		↓	↓			↓		↓		↓	↓	↑						↓	↓

N o t e: statistically significant changes of each parameter in comparison with the initial value for the given patient are shown (↓ reduction, ↑ increase).

T a b l e 3

Changes of biochemical parameters of detoxification in patients' blood serum after incubation with the cell culture

Parameters	Group "jaundice"									Group "cirrhosis"									
	P-3	P-5	P-7	P-11	P-12	P-13	P-15	P-17	P-20	P-1	P-2	P-4	P-6	P-8	P-10	P-14	P-16	P-18	P-19
Conjugated bilirubin (%): initial		55.2± 1.2	63.3± 0.9		60.9± 1.3		55.0± 0.5					37.0± 0.3	46.7± 0.4	56.1± 0.7				52.9± 0.9	33.6± 0.5
after incubation		56.1± 1.4	69.2± 1.2		66.2± 1.3		53.4± 0.5					40.1± 0.4	50.2± 0.5	60.2± 0.6				63.7± 0.8	39.3± 0.7
Unconjugated bilirubin (%): initial		44.8± 0.3	36.7± 1.2		39.1± 1.2		45.0± 0.5					63.0± 0.9	53.3± 0.3	43.9± 0.9				47.1± 1.2	66.4± 1.2
after incubation		43.9± 0.3	30.8± 0.6		33.8± 0.8		46.6± 0.4					59.0± 0.5	49.0± 0.9	39.8± 1.2				36.3± 0.8	60.7± 0.5
Total bilirubin		↓	↓		↓		↓					↓	↓	↓				↓	↓

N o t e: statistically significant changes of conjugated and unconjugated bilirubin levels (in percent of the total bilirubin) are shown for a given patient and of total bilirubin level in comparison with the initial value for the given patient (↓ reduction).
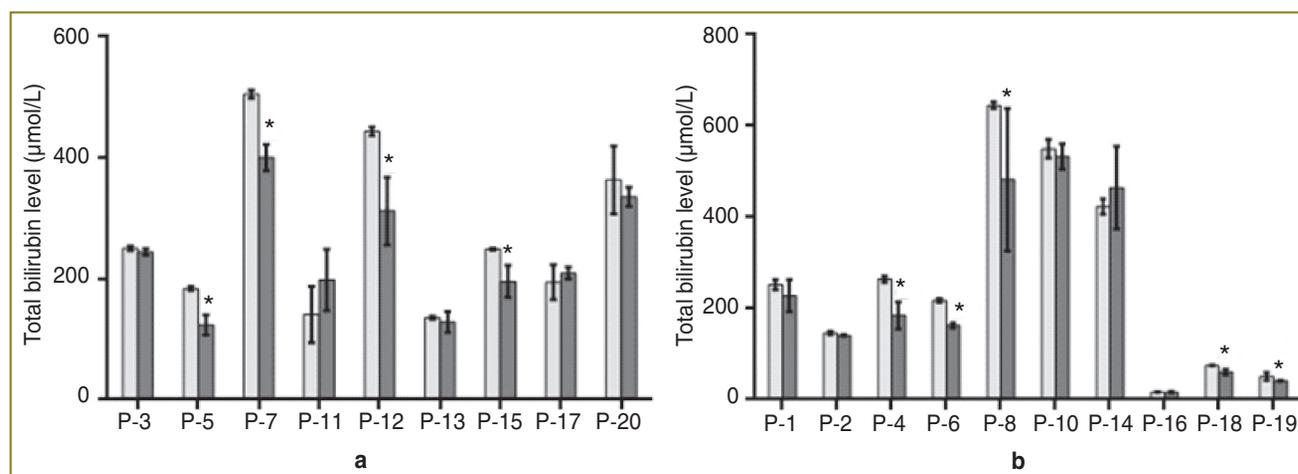
Figure 2. Change of the total bilirubin level in the blood serum of the patients from the group "jaundice" (a) and group "cirrhosis" (b) after incubation with the cell culture (light field — before exposure, dark field — after exposure); * statistically significant decrease of the total bilirubin level relative to the initial value in a given patient

was increased in sample P-6 (Table 2). Notably, a positive dynamics of a synthetic function was found in culturing the cells only with the patients' serum of group "cirrhosis", while in serum of group "jaundice" only statistically significant reduction of biochemical values of the synthetic function was observed.

Table 4

Changes of biochemical parameters of the cell destruction markers in the patients' blood serum after incubation with the cell culture

Parameters	Group "jaundice"									Group "cirrhosis"									
	P-3	P-5	P-7	P-11	P-12	P-13	P-15	P-17	P-20	P-1	P-2	P-4	P-6	P-8	P-10	P-14	P-16	P-18	P-19
AcAT	↑	↓	↓	↑	↓	↑									↑	↑	↑		
AIAT	↑			↑	↓	↑	↓	↓	↑	↑	↑				↑	↑	↑	↑	
LDG	↑	↑	↓	↑	↑	↑	↑		↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑

N o t e: statistically significant changes of every parameter level compared to the initial value for a given patient (↓ reduction, ↑ increase).
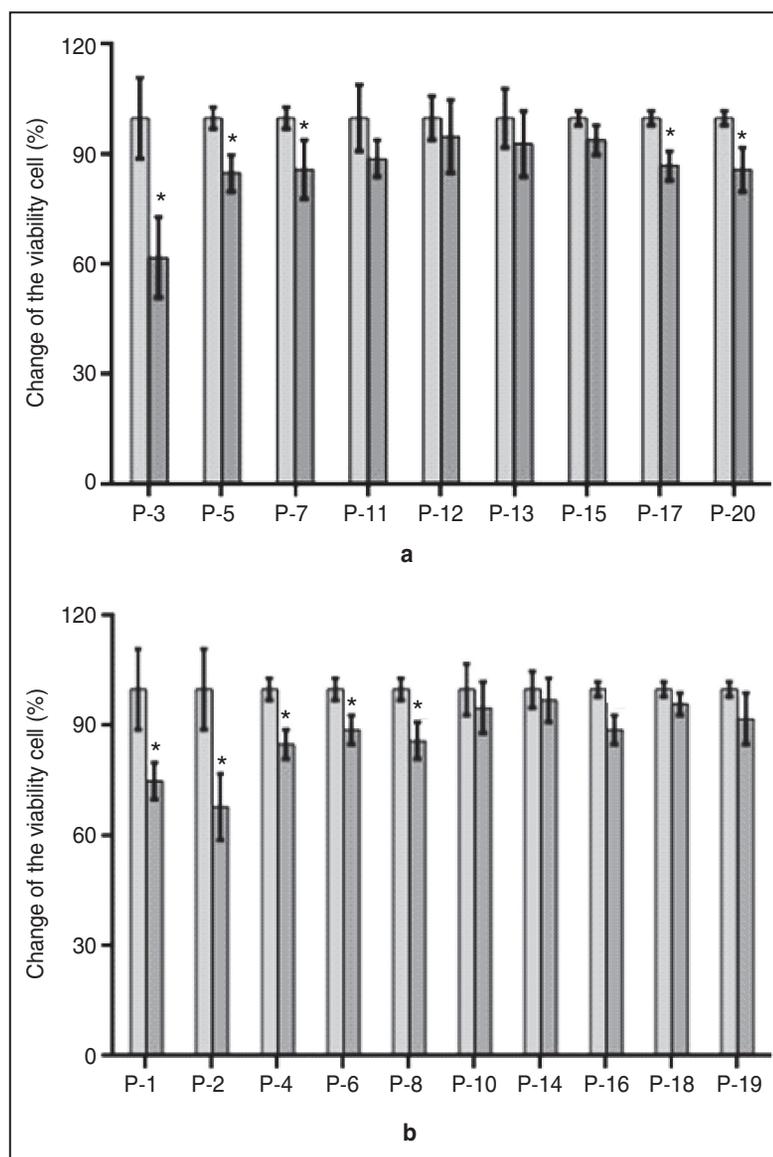
Figure 3. Change of the viability cell level after incubation with the blood serum of the patients from the group "jaundice" (a) and group "cirrhosis" (b) (light field — cell viability after incubation in the complete medium, dark field — cell viability after incubation in the patients' blood serum); * statistically significant decrease of the cell viability level

In relation to the detoxifying activity of Chang liver culture, statistically significant reduction of the total bilirubin level was established in 4 serum samples from group "jaundice" — P-5, P-7, P-12, and P-15. And in 3 samples (P-5, P-7, and P-15) positive alterations in the percent composition of its fraction were noted: simultaneous increase of conjugated bilirubin level and reduction of unconjugated one (Table 3). Similar dynamics of bilirubin fractions together with the reliable reduction of the total bilirubin level was noted in 5 samples from "cirrhosis" group: P-4, P-6, P-8, P-18, and P-19. Notably, the decrease of the total bilirubin level in the serum after the cell culture effect does not depend on the initial value of this parameter — the reduction occurs both in the samples with low values of the total bilirubin level (P-18 and P-19) and with the levels sharply exceeding the norm (P-7, P-8, and P-18) (Figure 2).

Biochemical components of the serum, in their turn, also influenced hepatocyte vital activity. Serum of the both groups were found to inhibit the life activity of the cells, and the most prominent negative effect was made by the serum of the "cirrhosis" group: the LDG level rose in the samples of all patients, the AIAT one increased in half the cases. Serum in the group "jaundice" did not affect significantly and negatively the cell culture (Table 4). Besides, the change of vital activity of the cells after their exposure to the serum samples of both groups was determined by MTT test (Figure 3). Statistically significant reduction of cell vital activity was established to occur after incubation with five of the nine samples from the "jaundice" group, and with six of the ten samples from the group "cirrhosis".

Discussion. Various models are designed to study the functioning of the cells in bioreactors, differing in the origin of hepatocytes (human immortalized, rats' primary), cultivation conditions (monolayer, spheroids on substrates, scaffolds), the origin of blood serum (human, porcine), and the time of cell culture exposure to the serum [17, 18]. The Chang liver cells, used in our work, has been traditionally applied as a hepatocyte-like test-system to determine cytotoxicity of various artificial and natural preparations [19, 20]. Some authors believe [14], that Chang liver line can also be used as a cell component in the artificial bioreactor systems. However, the aim of our investigation was to study the effect of Chang liver cell culture on the serum of patients with liver diseases of different etiology.

Group "jaundice" included patients with obstructive jaundice and developed hepatic failure of various degree. This condition is characterized by bile outflow impairment and deactivation of indirect bilirubin: the reduction of its elimination from the blood, derangement of its conjugation in the liver, and excretion in the form of direct bilirubin. It leads to the disturbances in the detoxifying function of the liver. Blood serum of such patients contain high levels of bilirubin and its fractions; destruction of hepatocytes results in the rise of transaminase concentration — AcAT, AIAT, and LDG as well. Besides, decrease of albumin content is observed due to the compromised synthetic function. Synthetic dysfunction can occur either as the result of cell function depression or decrease of the number of the functioning cells.

Group "cirrhosis" joined patients with Child–Pugh Class C cirrhosis, which are on a Waiting List for liver transplantation. Liver cirrhosis is a disease, characterized by focus necrosis of hepatic parenchyma with the following necrosis foci replacement by the connective tissue. Areas of regeneration from the functioning cells are not able to compensate the lost function of the hepatic parenchyma. Long course of the disease results in the development of hepatic failure in the form of synthetic and detoxifying damage of liver functions. In this case patient blood serum show significant reduction of bilirubin level, and lower values of bilirubin and its fractions compared to the group "jaundice".

In the majority of works, devoted to this topic, parameters of either synthetic (albumin, urea) [21] or detoxifying (ammonia) [22] function are used as a functional component of the effective work of the cell cultures. In our work complex evaluation of the biochemical parameters of the synthetic and detoxifying functions, exhibited by Chang liver culture, was used, being highly informative for the assessment of the effect of the cells on the blood serum, and determination of viability of the sells themselves. This model helped investigate the activity of the cell culture relative to the blood serum samples from the patients with hepatic failure.

Chang liver culture shows synthetic activity in the serum of the patients of the group "cirrhosis", while the serum of the group "jaundice" do not stimulate the synthetic function of the culture cells.

Detoxifying activity of the Chang liver culture, manifesting itself in the decrease of the total bilirubin level and positive dynamics of its fractions, is observed in both groups, but is most evident in the group "cirrhosis", as it occurs in half the cases. Serum is found to inhibit the viability of the cells in 50% of cases in each group.

Thus, the data obtained led to the conclusion, that Chang liver cell culture is most active in relation to the blood serum of the patients from the group "cirrhosis", where originally significant fall of albumin level and lower values of bilirubin and its fractions were observed.

Our results partially correlate with the data received by Nibourg et al. [23], investigating the effect of cytotoxic components of blood serum of the rats with induced acute liver failure on the cell line HepaRG in the model conditions of a bioreactor. The authors showed, that HepaRG cells are capable to perform a synthetic function (synthesis of urea, lipoproteins) being exposed to plasma, and also display the decrease of viability after 10 h of exposure.

Conclusion. The results of complex assessment of the main biochemical blood serum parameters can be used to determine the efficacy of application of various cell cultures as a model in the development of "bioartificial liver" systems, and for evaluation of the cell condition within a bioreactor. Immortalized Chang liver cell culture of hepatocytes is one of the candidates for the use in the composition of the artificial systems such as "bioartificial liver".

References

1. Stockmann H.B., IJzermans J.N. Prospects for the temporary treatment of acute liver failure. *Eur J Gastroenterol Hepatol* 2002; 14(2): 195–203, http://dx.doi.org/10.1097/00042737-200202000-00016.

2. Sauer I.M., Zeilinger K., Pless G., Kardassis D., Theruvath T., Pascher A., Goetz M., Neuhaus P., Gerlach J.C. Extracorporeal liver support based on primary human liver cells and albumin dialysis — treatment of a patient with primary graft non-function. *J Hepatol* 2003; 39(4): 649–653, http://dx.doi.org/10.1016/S0168-8278(03)00348-9.

3. Ryabinin V.E., Grobovoy S.I., Tkachev S.I., Kravchuk I.E. The study of hepatic cytosolic properties and the efficiency of its use in biological liver assist device. *Vestnik RAMN* 2002; 3: 21–24.

4. Ryabinin V.E., Suprun V.I., Tkachev S.I. *Ispol'zovanie iskusstvennykh sistem zhizneobespecheniya i kletochnykh tekhnologiy pri lechenii zabolevaniy pecheni* [Application

of artificial life support systems and cellular technologies in hepatotherapy]. Chelyabinsk: Yuzh.-Ural. nauch. tsentr RAMN; 2007.

5. Pan X.-P., Li L.-J. Advances in cell sources of hepatocytes for bioartificial liver. *Hepatobiliary Pancreat Dis Int* 2012; 11(6): 594–605, http://dx.doi.org/10.1016/S1499-3872(12)60230-6.

6. Priesner C., Hesse F., Windgassen D., Klocke R., Paul D., Wagner R. Liver-specific physiology of immortal, functionally differentiated hepatocytes and of deficient hepatocyte-like variants. *In Vitro Cell Dev Biol* 2004; 40(10): 318–330, http://dx.doi.org/10.1290/0404031.1.

7. Matsumura T., Takesue M., Westerman K.A., Okitsu T., Sakaguchi M., Fukazawa T., Totsugawa T., Noguchi H., Yamamoto S., Stolz D.B., Tanaka N., Leboulch P., Kobayashi N. Establishment of an immortalized human-liver endothelial cell line with SV40T and hTERT. *Transplantation* 2004; 77(9): 1357–1365, http://dx.doi.org/10.1097/01.tp.0000124286.82961.7e.

8. Sussman N.L., Chong M.G., Koussayer T., He D.E., Shang T.A., Whisennand H.H., Kelly J.H. Reversal of fulminant hepatic failure using an extracorporeal liver assist device. *Hepatology* 1992; 16(1): 60–65, http://dx.doi.org/10.1002/hep.1840160112.

9. Werner A., Duvar S., Müthing J., Büntemeyer H., Lünsdorf H., Strauss M., Lehmann J. Cultivation of immortalized human hepatocytes HepZ on macroporous CultiSpher G microcarriers. *Biotechnol Bioeng* 2000; 68(1): 59–70, http://dx.doi.org/10.1002/(sici)1097-0290(20000405)68:1<59::aid-bit7>3.0.co;2-n.

10. Hsieh S., Lin P.-Y., Hsieh C.-W., Li I-T., Hsieh S.-L., Wu C.-C., Huang Y.-S., Wang H.-M., Tu L.-W., Cheng K.-H., Wang H.-Y.J., Wu D.-C. Probing the adhesion of hepatocellular carcinoma HepG2 and SK-Hep-1 cells. *J Chin Chem Soc* 2012; 59(8): 929–933, http://dx.doi.org/10.1002/jccs.201200129.

11. Deurholt T., van Til N.P., Chhatta A.A., ten Bloemendaal L., Schwartlander R., Payne C., Plevris J.N., Sauer I.M., Chamuleau R.A., Elferink R.P., Seppen J., Hoekstra R. Novel immortalized human fetal liver cell line, cBAL111, has the potential to differentiate into functional hepatocytes. *BMC Biotechnol* 2009; 9: 89–104, http://dx.doi.org/10.1186/1472-6750-9-89.

12. Qiang Gao, Xiao-Ying Wang, Jian Zhou, Jia Fan. Cell line misidentification: The case of the Chang liver cell line. *Hepatology* 2011; 54(5): 1894–1895, http://dx.doi.org/10.1002/hep.24475.

13. Talbot N.C., Caperna T.J., Wells K.D. The PICM-19 cell line as an in vitro model of liver bile ductules: effects of cAMP inducers, biopeptides and pH. *Cells Tissues Organs* 2002; 171(2–3): 99–116, http://dx.doi.org/10.1159/000063704.

14. Yang T., Li C., Zhang L., Li M., Zhou P. A promising hepatocyte-like cell line, CCL-13, exhibits good liver function both in vitro and in an acute liver failure model. *Transplant Proc* 2013; 45(2): 688–694, http://dx.doi.org/10.1016/j.transproceed.2012.11.012.

15. Ellis F.J., Hughes R.D., Wendon J.A., Dunne J., Langley P.G., Kelly J.H., Gislason G.T., Sussman N.L., Williams R. Pilot-controlled trial of the extracorporeal liver assist device in acute liver failure. *Hepatology* 1996; 24(6): 1446–1451, http://dx.doi.org/10.1002/hep.510240625.

16. Török É., Vogel C., Lütgehetmann M., Ma P.X., Dandri M., Petersen J., Burda M.R., Siebert K., Düllmann J., Rogiers X., Pollok J.M. Morphologycal and functional analysis of rat hepatocyte spheroids generated on poly(L-lactic acid) polymer in a pulsative flow bioreactor. *Tissue Eng* 2006; 12(7): 1881–1890, http://dx.doi.org/10.1089/ten.2006.12.1881.

17. Hoekstra R., Nibourg G.A., van der Hoeven T.V., Ackermans M.T., Hakvoort T.B., van Gulik T.M., Oude Elferink R.P., Chamuleau R.A. The effect of rat acute-liver-failure plasma on HepaRG cells. *Int J Artif Organs* 2012; 35(11): 1006–1014, http://dx.doi.org/10.5301/ijao.5000121.

18. Lee J.-H., Lee D.-H., Park J.-K., Kim S.-K., Kwon C.H.D., Lee S.-K. Effect of fulminant hepatic failure porcine plasma supplemented with essential components on encapsulated rat hepatocyte spheroids. *Transplant Proc* 2012; 44(4): 1009–1011, http://dx.doi.org/10.1016/j.transproceed.2012.01.106.

19. Otang W.M., Grierson D.S., Ndip R.N. Cytotoxicity of three South African medicinal plants using the Chang liver cell line. *Afr J Tradit Complement Altern Med* 2014; 11(2): 324–329, http://dx.doi.org/10.4314/ajtcam.v11i2.16.

20. Trinh M.D., Ngo D.H., Tran D.K., Tran Q.T., Vo T.S., Dinh M.H., Ngo D.N. Prevention of H_2O_2-induced oxidative stress in Chang liver cells by 4-hydroxybenzyl-chitooligomers. *Carbohydr Polym* 2014; 103: 502–509, http://dx.doi.org/10.1016/j.carbpol.2013.12.061.

21. Yang Y., Li J., Pan X., Zhou P., Yu X., Cao H., Wang Y., Li L. Co-culture with mesenchymal stem cells enhances metabolic functions of liver cells in bioartificial liver system. *Biotechnol Bioeng* 2013; 110(3): 958–968, http://dx.doi.org/10.1002/bit.24752.

22. Guoliang L., Anye Z., Lifu Z., Xiaoping P., Yimin Z., Chengbo Y., Yuemei C., Lanjuan L. Effects of plasma from acute-on-chronic liver failure patients on immortalized human hepatocytes in vitro. *Hepatogastroenterology* 2011; 58(109): 1328–1333.

23. Nibourg G.A., Hoekstra R., van der Hoeven T.V., Ackermans M.T., Hakvoort T.B., van Gulik T.M., Chamuleau R.A. Effects of acute-liver-failure-plasma exposure on hepatic functionality of HepRG-AMC-bioartificial liver. *Liver Int* 2013; 33(4): 516–524, http://dx.doi.org/10.1111/liv.12090.